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16. ABSTRACT

A review of investigations utilizing electrophoretic and immunological methods for identification and classification of microsporidians, the group to which the first protozoan microbial pesticide belongs, indicate that these methods can be successfully used to classify strains and closely related species. Certain immunological methods are also useful for genera and families. The limitations and phylogenetic range of usefulness of other methods are discussed and suggestions made about future studies with Microsporida.

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MICROSPORIDIAN TAXONOMY: APPLICATION OF ELECTROPHORETIC AND IMMUNOLOGICAL TECHNIQUES[1]

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The phylum Microsporida is composed of a large group of diverse and ubiquitous eukaryotic species with intricate life cycles that multiply as obligate intracellular parasites. Partially as a reflection of this complexity, uncertainty surrounds their taxonomy. The properties and characteristics traditionally used in classifying these organisms appear not to have sufficed in producing a readily usable and discriminating system based on phylogeny. As a consequence the need for more definitive criteria for classification has been noted (Canning, 1977; Brooks, 1984).

In response to this need, a small number of investigators have begun to utilize alternative methods that have been in common use outside of microsporidiology. Essentially, these studies fall into two main categories based on the techniques utilized: (1) structural characterization by electrophoretic techniques, and (2) immunological characterization for grouping according to antigenic relationships.

To date most, if not all of the studies have been carried out with spores, the infective stage that facilitates their use as microbial pesticides (Canning, 1982). The reason for this frequent use of spores in investigations appears to be that purification is also expedited by the relative resistance of this stage to inactivation by environmental factors and their particulate nature. Additionally, spores predominate in moribund and dead individuals because it is the terminal stage of multiplication within a host. Thus spores are often available in reasonable quantities and biochemical and immunological characterization are possible. Such data have the potential to be utilized in microsporidian taxonomy. A side benefit of using the spore is that the techniques and assays that are developed may be applicable to field studies.

It is surprising that greater emphasis has not been placed on the evaluation of polyacrylamide gel electrophoretic and immunological methods as aids in deciphering microsporidian taxonomy. The results of the studies to date have all been encouraging in indicating their feasibility and utility to microsporidian classification. While space constraints do not permit a detailed review of the literature here, a brief summary follows.

[1]The research described in this paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

In general, the constitutive microsporidian spore proteins and their antigenic properties are fairly stable. Studies of hydrophobic protein extracts by gel electrophoresis (Fowler and Reeves, 1974a) and trichloroacetic acid precipitated whole spore homogenates of mono- and di-morphic species by sodium dodecyl sulfate gel electrophoresis (SDS PAGE) (Streett and Briggs, 1982a) indicate that neither host nor temperature of propagation affect the protein or polypeptide patterns obtained by gel electrophoresis. Time of harvest of the spores was also found to have no effect in the former study. Hydrophilic proteins, in contrast, appear to be more variable. These proteins were found *in toto* to be too variable to be very useful in gel electrophoretic analyses but selective utilization of the major bands appeared to be of possible value (Fowler and Reeves, 1974b). Hydrophilic proteins were also slightly affected in serological reactivity (Knell and Zam, 1978) with the development of partial identity in a minor precipitin band when a dimorphic species was grown at 21°C instead of 27° or 32°C. Similar results were observed between fresh and stored spores. Such effects, however, were considered minimal because they did not interfere with the differentiation of the cross-reacting species.

The results obtained with certain microsporidia used in common in a number of studies have been very consistent. For example, *Nosema bombycis* and *N. locustae* have been found to be unique in every electrophoretic (Streett and Briggs, 1982b; Langley, 1983) and immunological (Knell and Zam, 1978; Sato et al., 1981) study to date. Where comparative studies have been made of the species *Vairimorpha necatrix*, *V. plodiae*, and *V. heterosporum*, in both electrophoretic (Fowler and Reeves, 1974a; Streett and Briggs, 1982b) and immunological (Knell and Zam, 1978; Irby et al., 1986) analyses, these species have always been found to be closely related. *V. necatrix* and *V. plodiae*, have been shown to be nearly identical while *V. heterosporum* was more easily differentiable.

Many of these studies have also included new microsporidian isolates and in most cases the results permitted determination of the isolate's uniqueness or relationship to known species (Fowler and Reeves, 1974a; Streett and Briggs, 1982b; Streett and Henry, 1985; Knell and Zam, 1978; Sato et al., 1981; Irby et al., 1986). For example, Sato et al. (1981), were able to differentiate by immunofluorescence those microsporidian isolates from the silkworm that were related to *N. bombycis* from those that were not. Streett and Henry (1985) demonstrated the utility of SDS PAGE in determining that certain field isolates obtained from areas sprayed with *N. locustae* were distinct and represented a different species. The studies of Irby et al. (1986) showed the serological affinity of two dimorphic isolates to *Vairimorpha* species and the lack of relationship to *Nosema* species by immunoblot procedures in confirmation of the earlier SDS PAGE studies with one of the isolates (Bolivian) (Streett and Briggs, 1982b).

Several methodological developments have also occurred recently. Greenstone (1983) was able to develop an indirect enzyme-linked immunosorbent assay (ELISA) for spores of an *Amblyospora* species of *Culex salinarius* in order to screen individuals of scores of animal species that are possible intermediate hosts of the microsporidium. The lower level of sensitivity of the assay was reported as 3×10^3 spores.

Irby et al. (1986), utilizing the protein blotting technique (Gershoni and Palade, 1983), which is essentially a combination of SDS PAGE with ELISA, were able to visualize directly the antigenic relationships between species and ascertain the molecular masses of the separated exospore polypeptides that cross-reacted. The exospore proteins of microsporidia appeared to be

sufficiently diverse to provide a biochemical and immunological basis for the identification of microsporidia and potentially for the assessment of evolutionary relationships among species and genera. An indirect immunoferritin procedure, an ultrastructural equivalent of the fluorescent antibody technique initially used by Undeen and Alger (1977), was also utilized to demonstrate the location of spore surface antigens.

Langley (1983) used a two dimensional combination of isoelectric focusing and SDS PAGE (O'Farrell et al., 1977) to analyze the spore polypeptides present in homogenates of N. locustae, N. bombycis and V. necatrix. In this technique separation is effected in the first dimension according to the isoelectric points of the proteins and in the second dimension according to the molecular masses of the polypeptides. They found the two-dimensional "fingerprint" of each species unique and that nearly twice the number of polypeptides could be resolved with this method than with unidimensional SDS PAGE.

The utility of monoclonal antibodies with microsporidian spore polypeptides has been evaluated in our laboratory (unpublished observation). The results indicate that monoclonal antibodies with different specificities for spores of species other than the immunogen can be isolated. A comprehensive panel of monoclonal antibodies can potentially be utilized to characterize and delineate groups of related species as has been reported with bacteria (Conway De Macario et al., 1982). Monoclonal antibodies can also be utilized in sensitive ELISAs as has been done in our laboratory with spores of N. locustae.

CONCLUSIONS

In general, different methodologies utilized in classifying organisms have specific utility over characteristic ranges of phylogenetic relatedness. The two approaches that have been utilized most commonly with microspordian spore proteins, gel electrophoresis and immunological methods, are most effective over narrow phylogenetic distances. Electrophoregrams can establish phylogenetic relatedness (in contrast to compositional differences) between strains of species as well as closely related species while certain types of comparative immunological studies can provide data for establishing the affinities of species up to the level of family (Schleifer and Stackebrandt, 1983). While electrophoretic methods are faster and easier to conduct initially, immunological methods have the advantage over the long run because once antibodies are made to an antigen, partially purified or even crude cell-free extracts of other species can be used to carry out a large number of comparisons (Baumann et al., 1983). Monoclonal antibodies are especially suitable in this regard because large amounts of qualitatively identical antibodies can be produced indefinitely. This partially obviates the requirement for large numbers of spores for electrophoretic analyses. There is, however, a potential pitfall associated with serology that is especially relevant to studies with the microsporidia. Neiderkorn et al. (1980) have shown that sera produced to spores of Encephalitozoon cuniculi, an agent capable of producing chronic, subclinical infections of laboratory animals commonly used to produce antisera, cross react with many microsporidia including the entomophilic species N. algerae. Lack of appropriate controls such as preimmune serum in tests utilizing antisera from such animals could result in misleading data.

Nucleic acid hybridization techniques (DNA/DNA or RNA/DNA) are similar in range to electrophoretic methods. They are best applied to closely related species. For delineating even higher taxa, comparative studies

of the sequences of highly conserved molecules such as ribosomal RNAs appear to provide a good basis (Schleifer and Stackebrandt, 1983).

At this juncture of development, microsporidian taxonomy would be best served by the utilization of a variety of methods that are effective over differing ranges of phylogenetic relatedness. Those techniques that have been utilized to date have proven to be promising. Such data, when concordant, can be integrated with biological and ultrastructural information to provide, at the very least, an outline of the phylogeny of microspora.

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